• Basic Research •

Pharmacological drug screening to inhibit uveal melanoma metastatic cells either *via* EGF-R, MAPK, mTOR or PI3K

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Abstract

● **AIM:** To screen five potential pharmacological substances specifically targeting EGF-R, MAPK, mTOR, or PI3K for their antiproliferative effects, possible impact on cell viability, as well as cell death rates on three different uveal melanoma metastasis cell lines *in vitro*.

● **METHODS:** Three different uveal melanoma metastasis cell lines (OMM2.5, OMM2.3, and OMM1), that originated from human hepatic and subcutaneous metastasis, were exposed to inhibitors of different targets: erlotinib (EGF-R), everolimus (mTOR), selumetinib (MAPK), trametinib (MAPK) or the alkylphosphocholine erufosine (PI3K). Cell viability was assessed with a 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) dye reduction assay after 24h of treatment. Antiproliferative effects were evaluated separately after a 72-hour incubation of the cells with the pharmacological substance. Subsequently, the IC_{50} was calculated. Tumor cell death was investigated using a double stain apoptosis detection assay.

● **RESULTS:** Selumetinib, trametinib, and erufosine significantly decreased cell viability of all OMM cell lines (P<0.04). In addition, selumetinib and trametinib showed a significant inhibition of cell proliferation (P<0.05). Everolimus and erlotinib solely inhibited cell proliferation at the used concentrations (P<0.05). Besides an increase of necrotic cells after erufosine treatment (P<0.001), no changes in the number of dead cells for the other substances were observed.

● **CONCLUSION:** The preliminary drug screening demonstrates five new candidates, successfully targeting the canonical MAPK/ERK and PI3K/AKT/mTOR pathways in uveal melanoma metastasis cells *in vitro*. Hence, these findings provide an experimental basis to explore future single or combined therapy strategies for metastatic uveal melanoma.

● **KEYWORDS:** uveal melanoma; selumetinib; trametinib; erufosine; erlotinib

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INTRODUCTION

Uveal melanoma is the most common malignant ocular tumor entity. The incidence is estimated to be approximately 4-11 per one million citizens in developed countries per year, thus representing 5% of all malignant melanomas[1]. Unfortunately, the tumor is known to be aggressive and likely to metastasize with a survival probability of 57%[2]. Characteristic localizations of tumor spread are, in order of their probability of occurrence, the liver, the lung, the bones, and the skin. The occurrence of liver metastasis is crucial for the further course of disease as it typically results in a median survival of 6-12mo[3]. The development of metastasis located elsewhere offers a longer life expectancy[4].

Today, oncologists and ophthalmologists still lack a viable therapy for metastasized uveal melanoma. Different studies suggested combined therapies including dacarbazine as a potential drug for uveal melanoma metastasis treatment, which is already administered in metastasized cutaneous melanoma. However, its results are dissatisfactory as the mean survival did not increase significantly. In addition, the therapy frequently caused unfavorable side effects, such as diarrhea, fatigue, and nausea[5]. In the United States and

Europe, ipilimumab, a monoclonal antibody against cytotoxic T-lymphocyte-associated protein 4 (CTLA4), has been approved for the immunotherapy of advanced cutaneous melanoma. Unfortunately, in a phase II trial to investigate the effect of CTLA4 on metastatic uveal melanoma did not show encouraging results[6]. In line, the novel anti-programmed death (PD)1 antibody nivolumab could not enhance survival in uveal melanoma either[7].

Since the detection of GNAQ, GNA11, PTEN, and BRAF mutations in uveal and cutaneous melanoma, affecting subsequent signaling pathways such as the MAPK/ERK pathway and the PI3K/AKT/mTOR pathway, extensive development on treatment options for metastatic uveal melanoma has been performed. Inter alia, those pathways are activated by receptor-linked tyrosine kinases. Nevertheless, approaches to utilize the more unspecific tyrosine kinase inhibitors imatinib, sunitinib, sorafenib an others failed[8].

Therefore, there is a high demand to explore further pharmacological substances for the treatment of metastatic uveal melanoma: selumetinib and trametinib are inhibitors of the mitogen-activated protein kinase kinases MEK1 and MEK2[9], which are members of the MAPK/ERK signaling cascade[10]. Trametinib has been evaluated in a clinical study as a treatment for uveal melanoma[11], while selumetinib has already been considered in the use of metastatic uveal melanoma combined with dacarbazine[12].

Another possible drug is erlotinib, which mediates its therapeutic effects *via* inhibition of the tyrosine kinase domain of the epidermal growth factor (EGF) receptor. Erlotinib eventually leads to an inhibition of the canonical MAPK/ERK and PI3K/AKT/mTOR pathways, thus modulating cancer-modelling processes[13]. Currently, it is approved for the therapy of non-small cell lung cancer and pancreatic cancer[14].

Targeting the PI3K/AKT/mTOR pathway, as an inhibitor of mTOR, everolimus seems to be another promising candidate. It is inter alia used as a second-line therapy for advanced renal cell carcinoma[15].

Erufosine, an alkylphosphocholine, is a synthesized phospholipid-like molecule, exerting selective antitumor activity. A previous study proposes erufosine to mediate its anticancer effect primarily *via* inhibition of different members of the PI3K/AKT/mTOR signaling pathway, *e.g.,* for the therapy of oral squamous cell carcinoma[16].

The aim of our current study was to screen those five potential pharmacological substances for their antiproliferative effects on metastatic cells, possible impact on tumor cell viability, as well as tumor cell death rates on three different uveal melanoma metastasis cell lines.

MATERIALS AND METHODS

Cell Culture and Uveal Melanoma Metastasis Cell Lines For all experiments, three different uveal melanoma metastasis (OMM) cell lines were used. OMM2.5 and OMM2.3 originated from liver metastasis and were characterized by a Q209P in Guanine nucleotide-binding protein G(q) subunit alpha (GNAQ) mutation[17]. The third cell line, OMM1 was of subcutis origin and displayed a mutation of Q209L in GNA11. All cell lines were provided by Prof. Martine Jager (Department of Ophthalmology, Leiden University, Leiden, The Netherlands)[18].

Cells were cultured on uncoated cell culture dishes (NUNC, Langenselbold, Germany) in American Type Culture Collection (ATCC) modified Roswell Park Memorial Institute (RPMI) 1640 Medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 50 international units penicillin/mL and 50 µg streptomycin/mL (all obtained from Biochrom AG, Berlin, Germany). Cells were kept in an incubator at 37°C and 5% carbon dioxide.

Pharmacological Substances In total, 5 different pharmacological substances were tested in the experiments. All of them were obtained as powders from LC Laboratories (Woburn, MA, USA), except of erufosine, which was synthesized by Prof. Hans-Joerg Eibl (Max Planck Institute for Biophysical Chemistry, Goettingen, Germany). By dissolving in dimethyl sulfoxide (DMSO; Merck Millipore, Burlington, MA, USA), solutions of different concentrations (see below) of erlotinib, selumetinib, trametinib and everolimus were established and stored at 4°C until further utilization. Erufosine was dissolved in phosphate-buffered saline (PBS; Biochrom AG, Berlin, Germany).

Cell Viability of Treated Uveal Melanoma Metastasis Cells To evaluate, whether erlotinib, selumetinib, trametinib, everolimus or erufosine show any effects on the cell viability of the three cell lines, an XTT tetrazolium dye reduction assay [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; Thermo Fisher Scientific Inc.] and adapted to ophthalmologic cell culture[19], was performed. Concisely, cells were grown until a cell layer fully covered the cell culture plastic. Following incubation with serum-free cell culture medium for 24h, uveal melanoma metastasis cells were treated with various concentrations of erlotinib (25 nmol/L–100 µmol/L), selumetinib (1 nmol/L–200 µmol/L), trametinib (10 pmol/L–50 µmol/L), everolimus (100 fmol/L–100 µmol/L) or erufosine (10 nmol/L–199 µmol/L) for an additional 24h. An analogous amount of DMSO or PBS without a drug was added to the wells of the corresponding control group. Subsequently, the XTT assay was conducted in accordance to standard protocols. Finally, the absorption changes were measured at 450 nm using a spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, CA, USA).

Measurement of Antiproliferative Activity of Erlotinib, Selumetinib, Trametinib, Everolimus, and Erufosine on Uveal Melanoma Metastasis Cells Cell proliferation of uveal melanoma metastasis cells was measured using the XTT tetrazolium dye reduction assay as described above, however, with minor modifications. Cells were grown until they covered 15%–20% of the cell culture plastic and were kept in FBS-supplemented (10%) cell culture medium, to allow cell proliferation. Pharmacological treatment was conducted for 72h with varying concentrations of erlotinib (25 nmol/L–100 μmol/L), selumetinib (10 pmol/L–500 nmol/L), trametinib (10 pmol/L–5 μmol/L), everolimus (10 fmol/L–10 μmol/L) or erufosine (10 nmol/L–25 μmol/L). The same amount of DMSO or PBS used in the treatment groups was added to the corresponding control group. To determine the half maximal inhibitory concentration (IC_{50}) on cell proliferation on the three cell lines, a logistic regression fit was used.

Cell Death Determination of Uveal Melanoma Metastasis Cells under Pharmacological Treatment To assess apoptosis and necrosis rates in uveal melanoma metastasis cells, following treatment with the five pharmacological supplements, a double stain apoptosis detection assay (GenScript Biotech, Piscataway Township, NJ, USA) was performed. Cells of the cell lines OMM2.5, OMM2.3, and OMM1 were seeded onto slides with four incubation chambers (NUNC, Langenselbold, Germany) and prepared analogous to the cell viability assay. One chamber was used as an untreated control group. The cells of the other three chambers were each treated with a different concentration of erlotinib (25, 50, and 100 μmol/L), selumetinib (1, 10, and 100 μmol/L), trametinib (10, 100, and 50 μmol/L), everolimus (1, 10, and 100 μmol/L) or erufosine (10, 25, and 100 μmol/L). Following 24h of incubation, cells were stained with propidium iodide and Hoechst 33342 according to the manufacturer's instructions. Hoechst 33342 typically stains the chromatin of apoptotic cells brighter than normal cells, whereas propidium iodide is only permeant to dead cells. Microscopical images were acquired using a fluorescence microscope (Axio Observer 3, Carl Zeiss Meditec AG, Jena, Germany) and analyzed with the ZEN software (Carl Zeiss Meditec AG).

Statistical Analysis To compare the mean variables of more than 2 groups, an one-way ANOVA was performed. If the data met the criteria of the assumption of homogeneity of variances, a Bonferroni post-hoc test followed (SPSS 24.0; IBM, Armonk, NY, USA). *P*-values less than 0.05 were considered statistically significant. Data analysis was conducted with Microsoft Excel (Microsoft Office 365, Microsoft, Redmond, WA, USA). Graphs and logistic regressions were plotted with Prism 8 (GraphPad Software, San Diego, CA, USA).

RESULTS

Erlotinib Decreases Uveal Melanoma Metastasis Cell Proliferation The measurement of antiproliferative effects of erlotinib on OMM2.5, OMM2.3, and OMM1 cell lines revealed a decline in cell proliferation, starting at a concentration of approximately 1 μmol/L for all cell lines. A significant decrease of cell proliferation for OMM2.5 cells was observed at a concentration of more than 10 μmol/L by a maximum of 31% and at 5 μmol/L and above for OMM2.3 and OMM1 cells (by 29%–41%; for all $P<0.05$). The calculated IC_{50} was 3.83±4.35 μmol/L ($r^2=0.81$) for OMM2.5 cells, 4.51±0.87 μmol/L ($r^2=0.88$) and 7.11±2.78 μmol/L ($r^2=0.82$) for OMM2.3 and OMM1 cells, respectively (Figure 1A). For erlotinib, no cell viability changes in any of the three uveal melanoma metastasis cell lines were observed (Figure 1B). For all erlotinib concentrations tested, the double stain apoptosis detection assay did not show a significant difference of apoptotic cells compared to the control group (OMM2.5: $P=0.99$; OMM2.3: $P=0.95$; OMM1: $P=0.99$). In addition, no necrotic cells could be detected (Figure 1C).

Selumetinib Treatment at IC_{50} Inhibits Uveal Melanoma Metastasis Cell Proliferation Selumetinib mediated significant antiproliferative effects in OMM2.5, OMM2.3, and OMM1 cell lines at concentrations above 1 nmol/L ($P<0.04$) by 41%, 55%, and 54% respectively. The estimated IC_{50} was 5.01±2.05 nmol/L ($r^2=0.90$), 2.00±0.77 nmol/L ($r^2=0.86$) and 1.52±0.60 nmol/L ($r^2=0.92$) for OMM2.5, OMM2.3, and OMM1 cells, respectively (Figure 2A). Figure 2B indicates a significant reduction of uveal melanoma metastasis cell viability by a maximum of 52%–57% when compared to the untreated control group (for all groups: $P<0.04$). Interestingly, no difference in apoptosis rates (OMM2.5: $P>0.99$; OMM2.3: $P=0.92$; OMM1: $P=0.80$) and no necrotic cells for all tested concentrations and cell lines could be observed (Figure 2C).

Trametinib Affects Uveal Melanoma Metastasis Cell Proliferation and Viability For all three uveal melanoma metastasis cell lines, a significant inhibition by 41%-57% ($P<0.05$) of cell proliferation above a trametinib concentrations of 1 nmol/L could be observed (Figure 3A). Furthermore, according to the logistic regression, the IC_{50} was estimated to be 1.11±0.27 nmol/L ($r^2=0.93$) for OMM2.5, 2.30±0.65 nmol/L ($r^2=0.92$) for OMM2.3 and 2.27±0.44 nmol/L ($r^2=0.97$) for OMM1 cells (Figure 3A). As Figure 3B shows, trametinib caused a significant reduction of cell viability by 53%–61% at concentrations higher than 10 nmol/L in all cell lines (for all groups: $P<0.001$; Figure 3B). In consideration of apoptosis rates, for all concentrations, whether in OMM2.5 cells ($P=0.81$), nor in OMM2.3 cells ($P=0.94$), nor in OMM1 cells ($P=0.87$) significant differences compared to the untreated

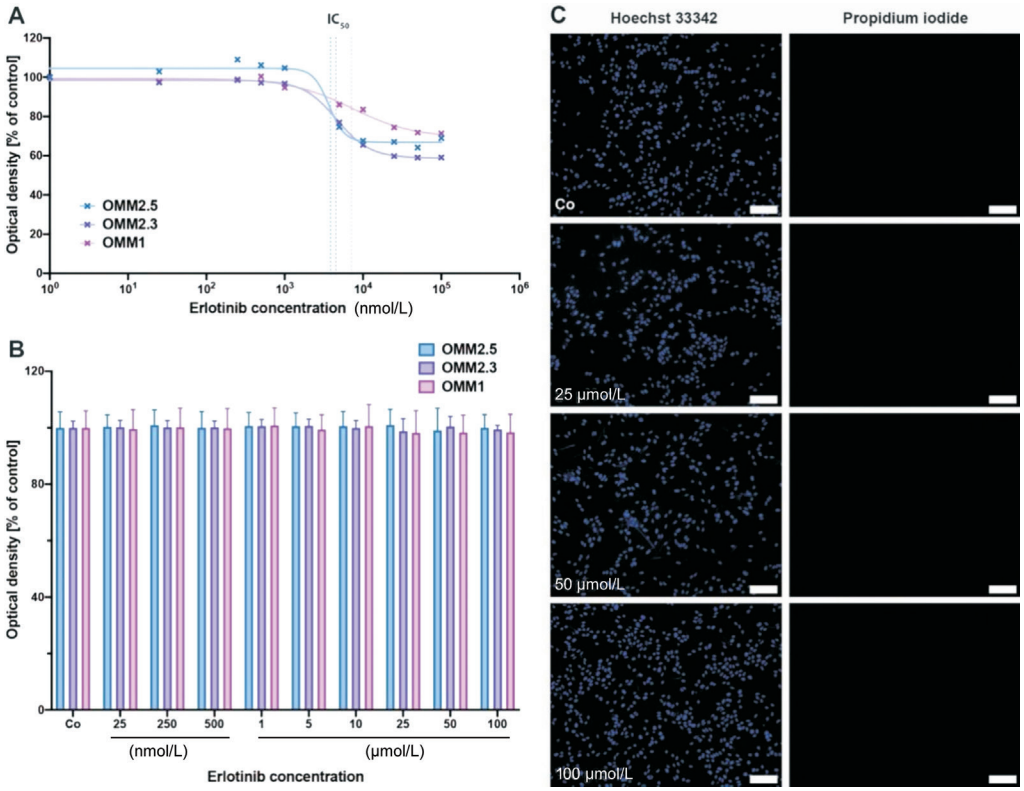

Figure 1 Erlotinib decreases OMM cell proliferation A: Cell proliferation of OMM cell lines after treatment with different erlotinib concentrations (n=4) and calculation of the IC_{50}; B: Cell viability following erlotinib treatment for 24h (n=4); C: Representative images of Hoechst 33342 and propidium iodide stained OMM2.5 cells (n=4 for each cell line and concentration). OMM: Uveal melanoma metastasis cell line; IC_{50}: Half maximal inhibitory concentration; Co: Control group. Scale bars: 100 μmol/L.

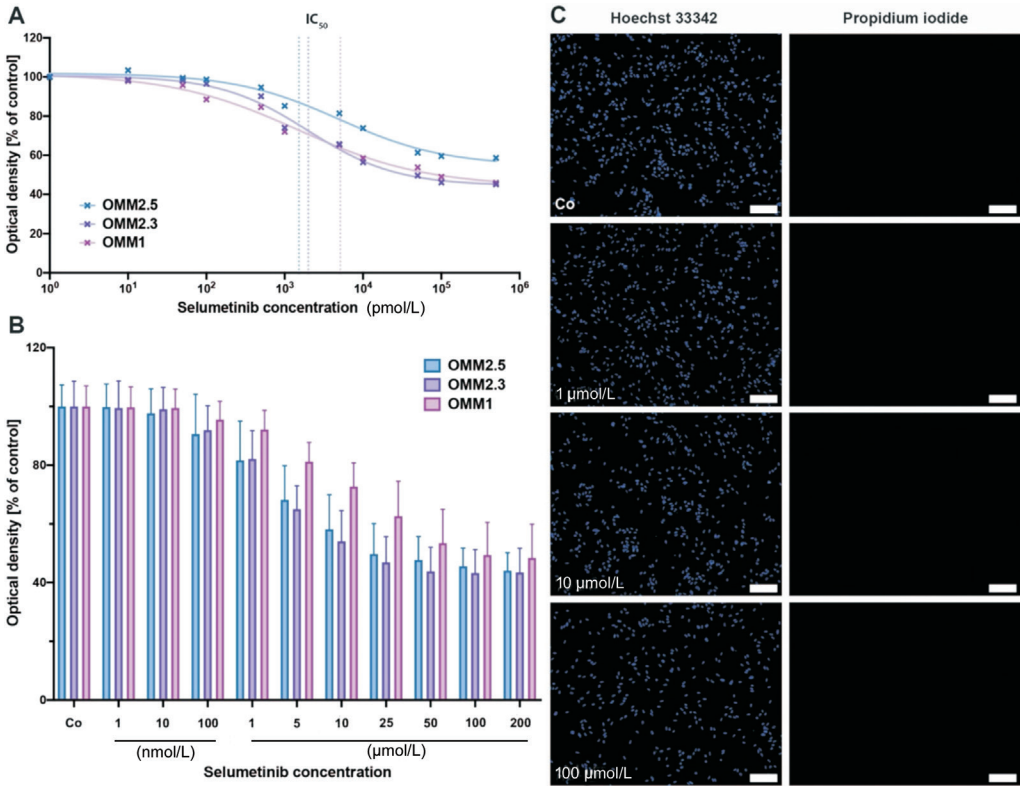

Figure 2 Selumetinib treatment inhibits OMM cell proliferation at IC_{50} A: Cell proliferation of OMM cells subsequent to 72-hour treatment with selumetinib (n=4) and IC_{50} calculation; B: Measurement of cell viability 24h after selumetinib treatment with different concentrations; C: Representative images of Hoechst 33342 and propidium iodide stained OMM2.5 cells (n=4 for each cell line and concentration). OMM: Uveal melanoma metastasis cell line; IC_{50}: Half maximal inhibitory concentration; Co: Control group. Scale bars: 100 μmol/L.

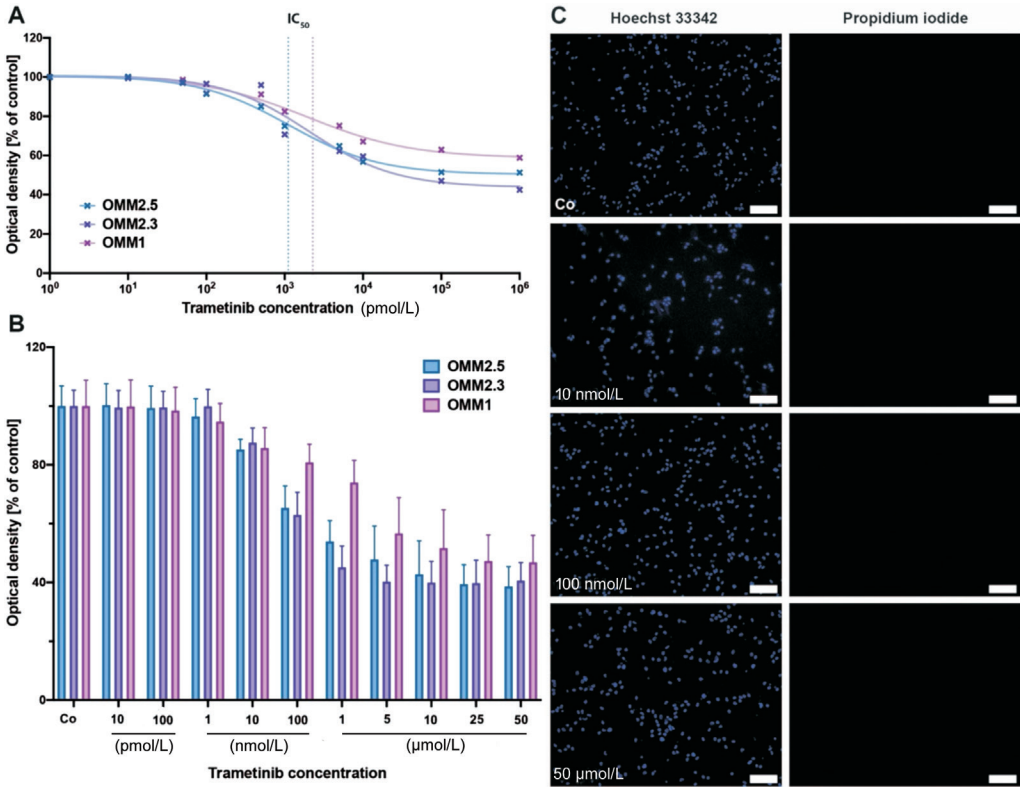

Figure 3 Trametinib affects OMM cell proliferation and viability A: Following 72h of trametinib treatment in different concentrations, cell proliferation was measured with an XTT assay (n=4) and the IC_{50} was calculated; B: Cell viability of OMM cells subsequent to 24h incubation with trametinib (n=4); C: Representative images of Hoechst 33342 and propidium iodide stained OMM2.5 cells (n=4 for each cell line and concentration). OMM: Uveal melanoma metastasis cell line; IC_{50}: Half maximal inhibitory concentration; Co: Control group. Scale bars: 100 μmol/L.

control group were detected. The propidium iodide staining did not reveal any necrotic cells (Figure 3C).

Uveal Melanoma Metastasis Cell Proliferation is Reduced by Everolimus A significant inhibition of uveal melanoma metastasis cell proliferation was observed at everolimus concentrations higher than 1 nmol/L (OMM2.3, OMM1) and 10 nmol/L (OMM2.5). Cell proliferation decreased by 34% for OMM2.5, by 51% for OMM2.3 and 38% for OMM1 cells. The logistic regression fit estimated the IC_{50} for OMM2.5 to be 4.09±1.39 nmol/L (r^2=0.90), 2.22±1.12 nmol/L (r^2=0.87) for OMM2.3 and 0.68±0.54 nmol/L (r^2=0.83) for OMM1 cells (Figure 4A). For all cell lines and concentrations, no decrease of cell viability could be observed (Figure 4B). No necrotic cells were found and no significant difference of the number of apoptotic cells between the treatment groups and the control group (OMM2.5: P=0.75; OMM2.3: P>0.99; OMM1: P=0.97) was detected (Figure 4C).

Erufosine Induces Necrosis Without Antiproliferative Effects As Figure 5A suggests, erufosine could not inhibit cell proliferation at any concentration on any of the three OMM cell lines. Thus, an estimation of the IC_{50} was not possible. Interestingly, a decreased OMM cell viability starting at concentrations of 10 μmol/L was observed for OMM2.5, OMM2.3, and OMM1 cells (for all groups: P<0.001; Figure 5B). Cell viability decreased to approx. The 5% for all cell lines when compared to the control group. In line, the number of necrotic cells of all concentrations and cell lines increased significantly (for all groups: P<0.001). Rising concentrations of erufosine increased the portion of necrotic cells compared to the overall detected cells in the corresponding Hoechst 33342-stained image to a maximum of 72%–79% (Figure 5C).

DISCUSSION

Our findings suggest that the inhibition of members of the MAPK/ERK and PI3K/AKT/mTOR pathways by the proposed substances, significantly reduced cell proliferation (selumetinib, trametinib, everolimus, and erlotinib), cell viability (selumetininb, trametinib, and erufosine) and induced cell necrosis (erufosine) in uveal melanoma metastasis cells *in vitro*.

Selumetinib and trametinib were able to inhibit cell proliferation, the root of a tumor spreading and metastasis growth, of all uveal melanoma metastasis cell lines significantly. Furthermore, both substances decreased cell viability significantly, which is another known target in cancer and metastasis treatment. As no necrotic cells could be observed, the anticancer effect of both MEK inhibitors seems to be mediated *via* apoptosis. This is in keeping with literature, presuming that trametinib causes a reduction of cell growth and

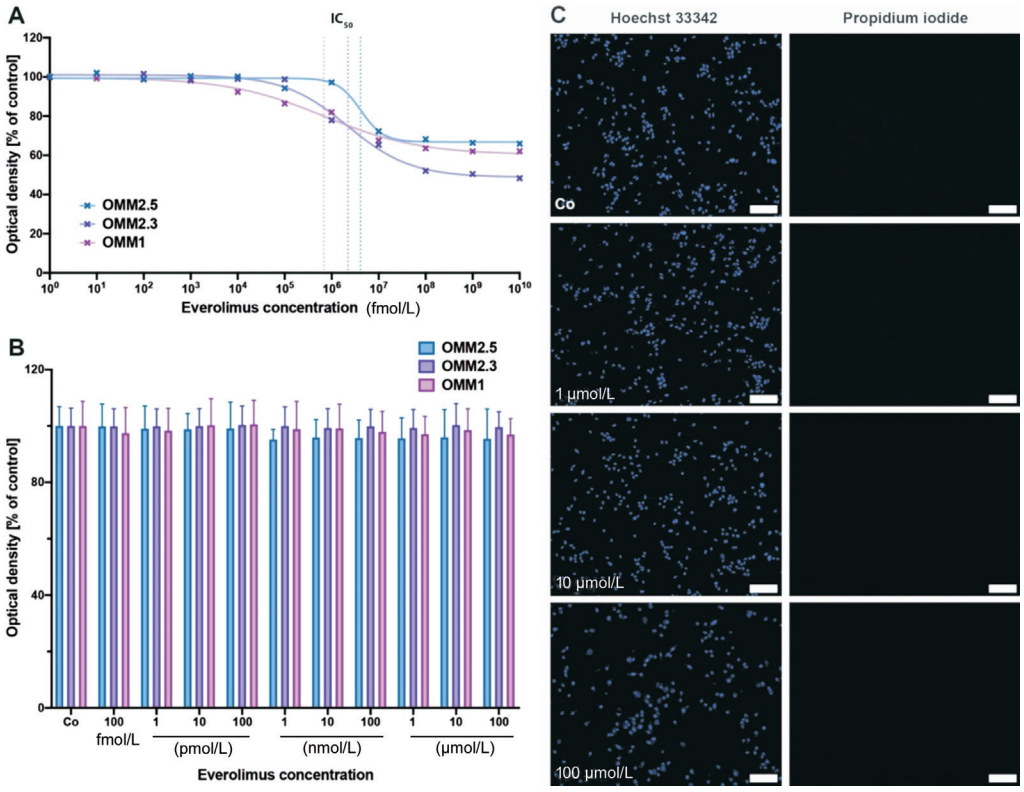

Figure 4 OMM cell proliferation is reduced by everolimus A: Cell proliferation of everolimus-treated OMM cells (*n*=4) and the subsequently calculated IC_{50}; B: Following 24h of OMM cell incubation with everolimus, cell viability was assessed using an XTT assay (*n*=4); C: Representative images of Hoechst 33342 and propidium iodide stained OMM2.5 cells (*n*=4 for each cell line and concentration). OMM: Uveal melanoma metastasis cell line; IC_{50}: Half maximal inhibitory concentration; Co: Control group. Scale bars: 100 µmol/L.

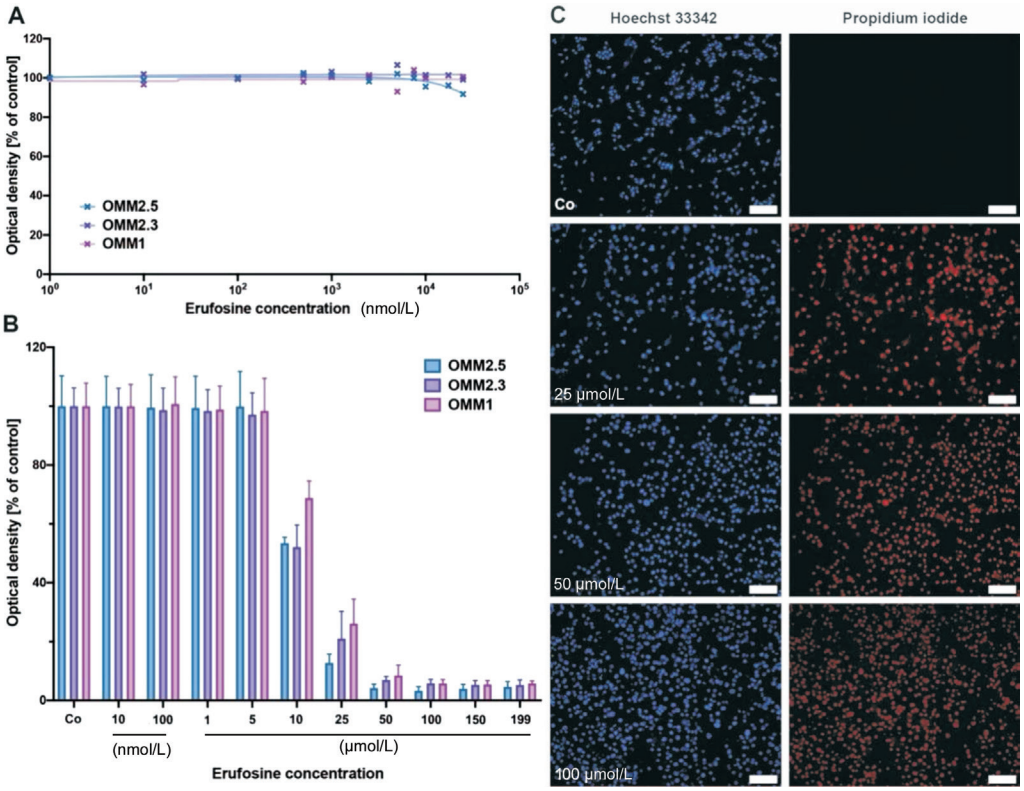

Figure 5 Erufosine induces necrosis without antiproliferative effects A: OMM cell proliferation was measured, using an XTT assay 72h after incubation with erufosine (*n*=4); B: Measurement of cell viability 24h after erufosine treatment with different concentrations (*n*=4); C: Representative images of Hoechst 33342 and propidium iodide stained OMM2.5 cells (*n*=4 for each cell line and concentration). OMM: Uveal melanoma metastasis cell line; Co: Control group. Scale bars: 100 µmol/L.

cell cycle arrest of uveal melanoma cells[9]. A study on GNA11-mutated cells treated with selumetinib showed a decrease of cell proliferation as well as cyclin D1 and increased levels of p27, which is considered a tumor suppressor and plays a major role in the signal transduction of apoptosis[20]. Furthermore, the investigations by Ambrosini *et al*[21] revealed an IC_{50} of less than 100 nmol/L to reduce cell viability of GNAQ-mutated cells. Those findings are in line with our results, and thus emphasize further preclinical exploration of the exact mechanism of action and optimization of the drug dose.

Erlotinib showed significant inhibitory effects on cell proliferation, even though the EGF-R mutation was not investigated. Due to the fact, that in some uveal melanomas, cell survival is mediated *via* receptor tyrosine kinases linked to the MAPK/ERK and PI3K/AKT/mTOR pathways, and erlotinib is known to inhibit the tyrosine kinase domain of the EGF receptor, it can be assumed that the EGF-R mutation potentially plays a role in uveal melanoma metastasis. Interestingly, Croce *et al*[22] found a few uveal melanomas to over-express EGF-R, which in turn raises the theory of EGF-R providing an additional anticancer target in metastatic uveal melanoma. Thus, we encourage a careful evaluation of EGF-R mutations in further studies on primary uveal melanoma metastasis cells. Moreover, studies on a safe human plasma concentration as well as the IC_{50} in rodents to inhibit the EGF receptor, proposed suitable concentrations of approximately 10 μmol/L[23], which is in line with our calculated IC_{50} for erlotinib.

Previously, for everolimus, an inhibition of cell proliferation on different uveal melanoma cell lines was shown *in vitro*. However, results were not significant, and the authors stated that the findings suggest a more cytostatic effect of everolimus[24]. This is in accordance with our measurements of a significantly decreased cell proliferation and unaffected cell viability. As everolimus is an mTOR inhibitor of the first generation, it only affects the protein complex mTORC1 and thus does not interfere with activation and inhibition of further targets of AKT[25]. This might be an explanation for the missing effects of everolimus on cell viability.

To our knowledge, the alkylphosphocholine erufosine has not been used in the context of uveal melanoma before. Theoretically, *via* interaction with the PI3K/AKT/mTOR and MAPK/ERK pathway, erufosine seems to be a promising candidate in metastatic uveal melanoma treatment. Nevertheless, we could not show an inhibition of cell proliferation in any cell line. *In vitro*, previous studies on head and neck squamous cell carcinoma[16] and non-small cell lung cancer cell line[26] suggested an IC_{50} of about 25 μmol/L. For primary chronic lymphocytic leukemia cells, the IC_{50} was calculated at 22 μmol/L, what is at the upper end of the concentrations used in our study[27]. Intriguingly, a significant reduction of cell viability and an increase of necrotic cells could be observed, implicating cytotoxic effects. Therefore, we conclude, that erufosine does not affect cell proliferation on uveal melanoma metastasis cells at the used concentrations. Still, this does not rule out antiproliferative effects at higher concentrations, that do not inhibit cell viability completely.

As the primary uveal melanoma can be treated satisfactorily, this study focused on the pharmacological drug screening for the treatment of metastatic uveal melanoma. The liver metastasis cells (OMM2.5 and OMM2.3) as well as the cutaneous metastasis cells (OMM1) showed typical mutations (Q209P in GNAQ or G209L in GNA11) for uveal melanoma and provide a well-established model for preclinical evaluation of pharmacological substances[24]. Nevertheless, this *in vitro* setting may encounter limitations. For example, when assessing apoptosis and necrosis counts, one has to consider that cell culture medium does not contain macrophages. Consequently, no phagocytosis of apoptotic cells takes place, leading to a secondary cellular leakage and may thus bias the apoptosis-necrosis-ratio. Furthermore, the exact mechanism and the specificity of some of the used substances is not yet completely understood. For example, a previous *in vitro* study on U0126, a highly selective MEK1 and MEK2 inhibitor revealed a remaining activity of the corresponding protein kinases of 56% and 92%, respectively[28]. Thus, due to a lack of specificity, expected results may fail to appear or significant unfavorable side effects can occur *in vivo*.

In summary, we present five drugs that either mediate a reduction of cell proliferation, cell viability or both of them in uveal melanoma metastasis cells *via* the MAPK/ERK and PI3K/AKT/mTOR pathways *in vitro*. It is our impression that those substances warrant further *ex vivo* and *in vivo* exploration as promising combined therapy options for metastatic uveal melanoma.

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Conflicts of Interest: Kassumeh S, None; **Arrow S,** None; **Kafka A,** None; **Luft N,** None; **Priglinger SG,** None; **Wolf A,** None; **Eibl-Lindner K,** None; **Wertheimer CM,** None.

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